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The development of induced pluripotent stem cell-derived mesenchymal stem/stromal cells from normal human and RDEB epidermal keratinocytes

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ABSTRACT

Background: Epidermolysis bullosa (EB) is a group of hereditary disorders caused by mutations in the genes encoding structural molecules of the dermal-epidermal junction (DEJ). Cell-based therapies such as allogeneic mesenchymal stem/stromal cell (MSC) transplantation have recently been explored for severe EB types, such as recessive dystrophic EB (RDEB). However, hurdles exist in current MSC-based therapies, such as limited proliferation from a single cell source and limited cell survival due to potential allogenic rejection.
Objectives: We aimed to develop MSCs from keratinocyte-derived induced pluripotent stem cells (iPSCs).
Methods: Keratinocyte-derived iPSCs (KC-iPSCs) of a healthy human and an RDEB patient were cultured with activin A, 6-bromoindirubin-3′-oxime and bone morphogenetic protein 4 to induce mesodermal lineage formation. These induced cells were subjected to immunohistochemical analysis, flow cytometric analysis and RNA microarray analysis *in vitro*, and were injected subcutaneously and intravenously to wounded immunodeficient mice to assess their wound-healing efficacy.
Results: After their induction, KC-iPSC-induced cells were found to be compatible with MSCs. Furthermore, with the subcutaneous and intravenous injection of the KC-iPSC-induced cells into wounded immunodeficient mice, human type VII collagen was detected at the DEJ of epithelized areas.
Conclusions: We successfully established iPSC-derived MSCs from keratinocytes (KC-iPSC-MSCs) of a normal human and an RDEB patient. KC-iPSC-MSCs may have potential in therapies for RDEB.

1. Introduction

Epidermolysis bullosa (EB) is a group of hereditary skin diseases characterized by widespread blister formation from birth. The recessive dystrophic EB (RDEB) subtype is caused by mutations in the *COL7A1* gene, which encodes type VII collagen, the main protein that forms anchoring fibrils beneath the dermal-epidermal junction (DEJ) [1–3]. There are no established therapies, only symptomatic treatments; therefore, extensive research has been conducted in search of effective treatments. Candidates for fundamental treatments are cell-based therapies, including allogeneic mesenchymal stem/stromal cell (MSC)-based therapies, as reported by several groups [4–6].

Abbreviations: α-MEM, alpha minimum essential medium; bFGF, basic fibroblast growth factor; BIO, 6-bromoindirubin-3′-oxime; BM-MSCs, bone marrow-derived MSCs; BMP4, bone morphogenetic protein 4; C57BL/6, C57BL/6 mouse; CM, conditioned medium; DAPI, 4′,6-diamidino-2-phenylindole; DEJ, dermal-epidermal junction; EB, Epidermolysis bullosa; EB-fibroblast, fibroblast derived from EB patient; EBKC, keratinocyte derived from EB patient; EBKC-iPSC, epidermolysis bullosa patient keratinocyte-derived iPSC; EBKC-iPSC-MSCs, MSCs derived from EBKC-iPSCs; EGF, epidermal growth factor; FABP4, Fatty Acid Binding Protein 4; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; PE, phycoerythrin; FITC, fluorescein isothiocyanate; HLA, human leucocyte antigen; iv, intravenous injection; iPSC, induced pluripotent stem cell; KC-iPSC, keratinocyte-derived iPSC; KC-iPSC-MSC, iPSC-derived MSC from human keratinocyte; LPE, local pooled error; MACS, magnetic-activated cell sorting; MEF, mouse embryonic fibroblast; MHC, major histocompatibility complex; MOI, multiplicities of infection; MSC, mesenchymal stem/stromal cell; NHEK, normal human epidermal keratinocyte; NHEK-iPSC, normal human epidermal keratinocyte-derived iPSC; NHEK-iPSC-MSCs, MSCs derived from NHEK-iPSCs; NOD/SCID, NOD/ShiJic-*scid*Jcl; RDEB, recessive dystrophic epidermolysis bullosa; sc, subcutaneous injection.

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MSCs are non-hematopoietic multipotent progenitor cells and heterogeneous populations of stromal stem cells [7]. MSCs can differentiate into cells of not only the mesodermal lineage, but also of ectoderm and endoderm lineages [8,9]. MSCs can differentiate into keratinocytes [8]. A number of studies have indicated that MSCs can significantly affect wound healing, through cell differentiation and the release of paracrine factors, implying a profound therapeutic potential [10–12]. Furthermore, it was reported that bone marrow-derived MSCs (BM-MSCs) supplement type VII collagen *in vivo*, which is one of the backgrounds to utilize allogeneic BM-MSCs for the treatment of RDEB [13]. However, there are some hurdles to overcome regarding treatments using MSCs for RDEB, such as limited proliferation from a single cell source and rather high invasion when donor MSCs are obtained from bone marrow for regenerative medicine.

To overcome these problems, we focused on the utilization of induced pluripotent stem cells (iPSCs). iPSCs have the ability to grow indefinitely while maintaining pluripotency [14]. We recently succeeded in establishing keratinocyte-derived iPSCs (KC-iPSCs) from a healthy human and RDEB patients with the Sendai virus vector (SVV) [15], for which keratinocytes can be obtained less invasively than by bone marrow harvest. It has been reported that the direction of differentiation of human iPSCs greatly depends on the origins of somatic cells [16], which is partly explained by the epigenetic memory of iPSCs [17]. Therefore, the ability of iPSCs in differentiation should be considered and investigated when the original somatic cells are different. It has been reported that fibroblast-derived iPSCs have the potential to convert to MSCs [18], but the generation of MSCs from keratinocytes-derived iPSCs has never been investigated.

In this study, we established MSCs from normal human and RDEB keratinocyte-derived iPSCs and analyzed their therapeutic potential as a strategy for the realization of *in vivo* iPSC-derived MSCs from human keratinocytes (KC-iPSC-MSCs) therapies.

2. Materials and methods

2.1. The patient

In this study, one patient with RDEB generalized intermediate was enrolled: a 58-year-old Japanese female who is compound heterozygous for c.5444 G > A and c.5819delC [19] in the *COL7A1* gene. This patient has recurring, widespread erosions and ulcers on the whole body (Fig. S1a). As a control, normal human epidermal keratinocytes (NHEKs) were cultured from the face skin of a healthy 67-year-old Japanese female volunteer. For immunofluorescence, skin samples from three healthy volunteers were used. This study was approved by the internal review board on ethical issues of Hokkaido University Hospital (approval #014-0041). Written informed consent was obtained from the participants before the processes conducted in this study.

2.2. The mice

C57BL/6 mice (C57BL/6) and NOD/ShiJic-*scid*Jcl (NOD/SCID) mice were purchased from CLEA Japan (Tokyo, Japan). They were handled according to guidelines provided by the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol (approval #15-0158).

2.3. The cells

To obtain NHEKs and keratinocytes derived from EB patient (EBKCs), we harvested full-thickness intact skin specimens under local anesthesia. The samples were immersed in dispase (0.04 mg/ml, Sigma-Aldrich, St. Louis, MO) solution for 16 h and we separated the epidermis from the dermis mechanically with tweezers. We minced the epidermis into small pieces using scissors and put the pieces into a 15 ml conical tube (Thermo Fisher Scientific, Waltham, MA) containing 2 ml of 0.25% trypsin and incubated at 37 °C for 7 min. Then, we neutralized the trypsin using 6 ml of phosphate-buffered saline (PBS) containing 10% fetal bovine serum (FBS, Sigma-Aldrich), and centrifuged it at 300xg for 5 min at 25 °C. The medium was aspirated, and the cell pellet was resuspended in the desired volume of CnT-PR medium (CELLnTEC, Bern, Switzerland). These cells were seeded at 5,000–10,000 cells/cm^2 and placed in a humidified incubator at 37 °C and 5% CO_2. The medium was replaced one day after plating and again every 2–3 days until 70–80% confluence. We used these keratinocytes at passage 3 to 4 for further experiments.

Keratinocyte-derived iPSCs (KC-iPSCs) were established using the following: SVVs encoding four Yamanaka factors (*OCT3/4*, *SOX2*, *KLF4* and *c-MYC*; CytoTune®-iPS 2.0; ID Pharma, Tokyo, Japan) as we reported previously [15], NHEK-derived iPSCs (NHEK-iPSCs) and EBKC-derived iPSCs (EBKC-iPSCs). The keratinocytes were seeded at 50,000 cells/cm^2 with CnT-PR medium. The next day, keratinocytes were transduced by SVVs at multiplicities of infection (MOI) = 5. After 6 days, the transduced cells were reseeded on mitomycin C (MMC)-treated mouse embryonic fibroblasts (MEFs, ReproCELL, Yokohama, Japan), which were seeded at 30,000 cells/cm^2 in advance. The next day, the medium was changed to human embryonic stem cell medium (Primate ES Cell Medium, ReproCELL). When small, sharp-edged, tightly packed iPSC-like colonies emerged two weeks after SVV transduction, individual colonies were chosen. KC-iPSCs were cultured on MEFs in embryonic stem cell medium containing 5 ng/mL basic fibroblast growth factor (bFGF, ReproCELL) in a humidified incubator at 37 °C and 5% CO_2. The medium was changed daily. iPSC-like colonies without substantial spontaneous differentiation were obtained between 2 and 10 clones per 10^5 transduced keratinocytes. We used these KC-iPSCs at passages 8–25 for further investigations.

BM-MSCs as controls were purchased commercially (Lonza, Basel, Switzerland), and these were expanded in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) containing 10% FBS and used at passages 4–6. Fibroblast-derived iPSCs were purchased from the RIKEN BioResource Center (RBRC-HPS0063, Tsukuba, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan, as a positive control of iPSCs [14]. Morphological shapes were recorded with DP Controller and DP Manager (Olympus, Tokyo, Japan).

2.4. The derivation of mesenchymal stem cells from KC-iPSCs

We performed the differentiation of KC-iPSCs into MSCs according to the protocol previously described by Tran and colleagues [18] (Fig. 1a). NHEK-iPSCs (passages 21–25) and EBKC-iPSCs (passages 8–10) were used for the derivation into KC-iPSC-MSCs. Two cell lines were chosen for derivation into KC-iPSC-MSCs in each iPSC. These cells were cultured on MMC-treated MEFs in human embryonic stem cell medium containing 5 ng/mL bFGF in a humidified incubator at 37 °C and 5% CO_2. For feeder-free cultures of KC-iPSCs, we used MEF-conditioned medium (CM), which has previously been reported [20]. KC-iPSCs were first transferred into Matrigel® (Matrigel Basement Membrane Matrix, Corning, New York, NY)-coated dishes in MEF-CM containing 10 ng/mL bFGF and were then stabilized in an atmosphere of 5% CO_2 at 37 °C for 2 days. Second, for induction into mesoderm-lineage cells, KC-iPSCs were incubated in ES medium containing 5 ng/mL Activin A (Peprotech, Rocky Hill, NJ), 2 mM 6-bromoindirubin-3′-oxime (BIO, Sigma-Aldrich), and 20 ng/mL bone morphogenetic protein 4 (BMP4, Peprotech) for

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